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Screening of different thermal receipt papers for their potential endocrine activity

영수증의 내분비계 교란 스크리닝 연구

2017년 8월

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이 논문을 보건학석사 학위논문으로 제출함

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Abstract

Screening of different thermal receipt papers for their potential endocrine activity

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Bisphenol A (BPA), a weak estrogenic chemical, is still widely used in industrial and consumer applications. Oral ingestion of BPA via contaminated food results in major BPA exposure for the most part, however contact to thermal receipt papers contributes to major non-dietary exposure to this important chemical. BPA is used as a color developer on a thermal receipt paper in color development process. BPA has endocrine disruptive effects; therefore, structural or functional alternatives to BPA have been replaced and manufactured. However, we have limited information on their potential adverse effects. In this study, we obtained three kinds of thermal receipt papers, i.e., BPA, BPA free, non-bisphenol thermal receipt papers, and screened for their potential endocrine effects. The extracts of three receipt papers were performed on GH3 and H295R cell lines to investigate their possible disruptive effects in thyroid or sex hormone systems. The amount of color developer on thermal receipt paper is equivalent to 1 % of total receipt paper weight. Both cell lines were exposed to the extracts with 0, 0.0015, 0.005, 0.015, 0.05, or

0.15 mg developer/g receipt paper in 0.1 % ethanol of cell medium. In GH3 cells, BPA and BPA free thermal receipt extracts reduced thyroid stimulating hormone (*Tsh)* gene expression level significantly. However, other thyroid hormone related genes such as *Trα*, *Trβ*, *Dio1*, and *Dio2* did not showed any down- or up-regulations after the exposure. Those two extracts also reduced transcriptional levels in follicular stimulating hormone (*Fsh*), estrogen receptor α (*Erα*), and β (*Erβ*). Non-bisphenol thermal receipt paper extract did not show any alteration in both thyroid and gonad axis related genes. In H295R cell lines, all three thermal receipt paper extracts increased in production of 17β-estradiol at their highest concentrations (0.05 and 0.15 mg/g receipt paper). However, they did not lead to any changes in testosterone level and related gene transcription levels. In this study, all three investigated thermal receipt papers showed endocrine disruption potential on either thyroid or sex hormone system. The results of this study shows that BPA free and non-bisphenol thermal receipt papers may not be safer alternatives to BPA thermal receipt paper. This study showed possible toxic effects from all three extracts of thermal receipt papers. Further studies should follow to evaluate the toxicities of BPA free receipt paper *in vivo* to develop better substituting chemicals for color development process in receipt paper.

Keywords: Thermal paper, receipt paper, BPA, alternatives, BPA free, color development, thyroid hormone, sex hormone

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1. Introduction

Bisphenol A (BPA) is a monomer and widely applied to many industrial and consumer products (Vandenberg et al., 2010). Worldwide production of BPA has been grown steadily from 0 % to 5 % annually (Burridge, 2008). BPA has been used in poly carbonate plastics such as bottles, tableware, and food-packaging materials (Liao and Kannan, 2011) and these applications have been recognized as the major routes for human exposure (Geens et al., 2012). Among the non-dietary routes, the skin absorbance is a major pathway of human exposure from contacting thermal papers (von Goetz et al., 2017). Recent reports from different countries pointed out that BPA had been detected frequently and quantitatively in various thermal papers including point of sale receipts, parking tickets, bank receipts, and luggage tag (Vinggaard et al., 2000; Östberg T, 2010; Liao and Kannan, 2011; Goldinger et al., 2015; Rocha et al., 2015; Russo et al., 2017). BPA forms a color-developing complex by reacting with leuco dye under heat or pressure (Geens et al., 2012). BPA presents non-polymerized form on the thermal paper, which makes BPA easily transfer from paper to other contacted materials (Terasaki et al., 2007). Human exposure level from contacting thermal receipt paper is detectable and the maximum calculated estimated daily intake (EDI) for occupational exposure level was 218.3 μg/day in China (Fan et al., 2015). Notably, BPA is transferred to the fingers by holding the thermal receipt paper in 5 s (Biedermann et al., 2010). Several studies have also reported that the frequency of contacting thermal receipt paper has an association with the

amount of BPA found in the urine (Hehn, 2015; Ndaw et al., 2016; Thayer et al., 2016).

The application of alternatives has been expanded to reduce the exposure to bisphenol A. Recently, EC proposed a draft to set a limit of BPA application to thermal paper to 0.02 % in weight in thermal paper (Bjornsdotter et al., 2017 b). The US Environmental Protection Agency propounded a list of 19 candidates for color developing compounds in thermal paper (US Environmental Protection Agency, 2015). Due to lack of toxicity data, it is hard to assume that the alternatives are safer replacement for BPA. Recent studies have been reported high detection frequency of bisphenol S (BPS), a popular alternative color developer (Liao et al., 2012; Goldinger et al., 2015; Thayer et al., 2016; Russo et al., 2017). BPS bonded to the estrogen receptor (ER) (Yamasaki et al., 2004) and induced MCF-7cell proliferation (Kuruto-Niwa et al., 2005). Moreover, BPS has been reported as thyroid hormone system disruptor by altering the whole-body thyroid hormone levels in zebrafish larvae (Zhang et al., 2017). The detection amount and frequency are very little but other structural or functional analogues (Pergafast 201, D-8, D-90, TGSA, and PBS-MAE) were reported recently (Bjornsdotter et al., 2017 b).

Formally, several papers have reported detection of color developers in thermal receipt papers and tested their estrogenic activity and developmental effects employing *in vitro* or *in vivo* methods, respectively (Goldinger et al., 2015; Bjornsdotter et al., 2017 b). Goldinger et al. (2015) analyzed 124 thermal paper receipts and targeted to detect only color developers (BPA and

its alternatives) which were further exposed to H295R cells to exam their steroidogenic effects. Bjornsdotter et al. (2017 b) tested color developers detected from receipt papers on cells and zebrafish embryo to observed estrogenic activity and teratogenic effects, respectively. The color developer is suspected as the potential toxic inducing compound among the thermal paper components. However, the thermal receipt paper is composed of different compounds such as color former, pigment, binder, and color developer (Lassen and Brandt, 2011). Therefore, the assessment of the thermal receipt paper needs to be expanding to the whole paper extract toxicity test.

Bradley et al. (2008) conducted a safety assessment of paper and board used for food contact containers. The group employed several solvents (water, ethanol, and tenax) for extraction. Water based extracts had pulp-derived substances while ethanol based extracts had organic compounds mainly. This study extracted the sample thermal receipt papers with 10% ethanol. The extract tentatively contains both pulp and organic components such as leuco dye, color developers, sensitizers, and stabilizers. It is hard to distinguish single chemical adverse effects on the organism for exposing the paper extracts. However, this extracts were subjected to screen endocrine disruption effect by conducting effect driven analysis. This study has started with suspecting BPA and its alternatives as the major toxic compounds on the thermal paper.

We have limited knowledge of toxic potential of BPA applied on thermal receipt paper. On the other side, alternatives, chemically and/or functionally similar to BPA, have potential toxic effects, but they are unknown. This study is performed to

investigate potential endocrine disrupting effects of commercially available thermal receipt papers using test systems sensitive to propose adverse effects on thyroid system or reproduction system.

Table 1. Thyroid hormone and sex hormone disrupting effects of BPA and BPS *in vitro*

System	Chemical	Model	Endpoint(s)	Concentration	Result	Reference
Thyroid hormone	BPA	GH3	Thyroid receptor	0.01, 0.1, 1, 10 mg/L	*Tshβ, Trα, Trβ, Dio1, Dio2*↓	Lee et al., 2017
	BPA	FRTL-5	Thyroid hormone synthesis	0, 1, 10, 100 mg/L	*Tshr, Pax8, Nkx2.1, Nis, Tg, Tpo* ↑	Lee et al., 2017
	BPA	FRTL-5	Hene expressions related to thyroid hormone synthesis	0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M	*Nis, Tshr, Tpo, Tg, Pax8, Nkx2.1* ↑	Gentilcore et al., 2013
	BPS	GH3	Thyroid receptor	0, 0.1, 1, 10, 100 mg/L	*Tshβ, Trα, Trβ, Dio1, Dio2*↑	Lee et al., 2017
	BPS	FRTL-5	Thyroid hormone synthesis	0, 0.1, 1, 10 mg/L	*Tshr, Pax8, Nkx2.1, Nis, Tg, Tpo* ↑	Lee et al., 2017
Sex hormone	BPA, BPS	H295R	Steroidogenesis	0, 0.1, 0.3, 1, 3, 10, 30, 100 μM	BPA, 17-βestradiol level↑ BPA and BPS, Testosterone hormone ↓	Goldinger et al., 2016
	BPA, BPS	H295R	Steroidogenesis	0, 0.1, 1, 10, 30, 50 μM	BPA and BPS were capable of altering Steroidogenesis in H295R cells. BPA and BPS, Testosterone hormone ↓	Feng et al., 2016
	BPA	H295R	Steroidogenesis	0, 0.0098, 0.093, 0.156, 0.365, 2.50, 10 μM	Testosterone hormone ↓	Zhang et al., 2012
	BPA	MCF-7	Genotoxic and cytotoxic	0, 0.4, 1, 4, 40, 100 mg/L	Chromosomal aberrations in ER-dependent pathway	Aghajanpour-Mir et al., 2016

2. Materials and Methods

2.1. Chemicals

Ethanol (purity 99.8 %, Daejung Chemicals) was purchased and used as solvent in all test methods.

2.2. Thermal receipt papers and sample preparation

Samples of unprinted thermal receipt papers with different color developers were obtained from online market. The thermal receipt papers were cut into strips using paper shredder and stored in a sealed poly bags. Each thermal receipt paper was weighed for 1.5 g and immersed in 10 mL of 10 % ethanol solution based cell media. The extraction was continued by 1 minute of gentle vortex followed by 15 minutes of sonication at room temperature. The final extract was diluted with cell media or dilution water with maintaining 0.1 % (v/v) ethanol. The concentration of color developer was calculated based on Mendum et al. (2011) study that BPA concentration in receipt considered as 1 % in 100 g receipt paper.

2.3. GH3 cell culture and exposure

GH3 cells are rat pituitary tumor cells. GH3 cells were cultured in a Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma Aldrich) with 10 % fetal bovine serum (FBS; Wellgene, Korea) and treated in 100 mm cell culture dishes at 37 ℃ in a humidified atmosphere with 5 % CO_2. GH3 cells were

seeded in 96-well plate in density of $2.5x10^5$ cell/well for preliminary range finding test. The growth medium was replaced with serum-free medium supplemented with 1 % BD ITS + premix (BD Biosciences, Franklin Lakes, NJ, USA) after 20 h of initial seeding in order to make free status of steroid hormones and gro wth factors in the GH3 cells. After four hours of media replacement, cells were exposed to each thermal receipt extractions with different concentrations for 48 h in quintuplicates (N=5). At the end of the treatment, cell proliferation was measured using a WST-1 cell proliferation assay (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol. The selected concentrations were applied to GH3 cells for analyzing transcriptional changes in GH3 cells from exposure to each thermal paper extracts. Test procedure was same as cell proliferation test as describe above, but 24-well plate was used in this test (3 replicates). 3,3'5-triiodo-L-thyronine (T3, Sigma Aldrich, St. Louis, MO, USA) will be used as a positive control at concentration of 0.0065, 0.065, and 0.65 μg/L.

2.4. H295R cell culture and exposure

H295R cells are human adrenal carcinoma cells. Cells were cultured in a mixture of Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma Aldrich) with addition of 1 % TIS + Premix (BD Bioscience Franklin Lakes, NJ, USA), 2.5 % Nu-Serum (BD Biosciences). The incubator environment kept 37 ℃ in a humidified atmosphere with 5 % CO2 conditions. The exposure concentrations are going to be selected based on > 50 % cell viability from WST-1

cell cytotoxicity assay. For the cell bioassay, the cells were seeded into 24-well plate in density of $3.0x10^5$ cell/well. The cells were dosed with different concentrations of thermal receipt paper extraction. Forskolin (an inducer of steroid hormone production) used as positive controls. The exposure was performed for 48 h with three replicates. At the end of exposure, cell medium were collected for sex steroid hormone measurement, and cells for gene transcription analysis.

2.5. Measurement of sex hormones

H295R cell sex steroid hormones were extracted with diethyl ether and measured by ELISA using commercially available kits (Cayman Chemical; 17ß-estradiol [Cat No. 582241], and testosterone [Cat No. 582701]). The intra-and inter-assay coefficients of variation (CV) were ≦ 30 %. Briefly, 500 μL of H295R cell medium was diluted with 400 μL of Ultrapure water, then extracted with 2.5 mL of diethyl ether by centrifuging at 2100 rpm for 10 min at 4 ℃. The extraction step was repeated twice. Once the diethyl ether evaporated, the sample was reconstituted in 300 μL of EIA buffer provided from ELSA assay.

2.6. Measurement of transcriptional changes

Total RNA in cells were extracted using RNeasy mini kit (Qiagen) according to manufacturer's instruction. Quality and quantity of RNA were checked with an Epoch Take 3 microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany). Complementary DNAs (cDNAs) were synthesized from the total RNA (100 ng/μL)

using the iScript cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). Quantitative real-time PCR (RT-PCR) was performed with LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK) using LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA). The thermal cycle profile was: preincubation at 95 ℃ for 10 min, 40 cycles of amplification at 95 ℃ for 10 s, 85 ℃ for 20 s, and 72 ℃ for 20 s. For quantification of PCR results, the threshold cycle (Ct) will be determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene (*Cyclophilin* amd *β-ACTIN* for GH3 cell and H295R cell, respectively) by the use of the2- ΔΔCt method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Statistical analysis was performed using SPSS 23.0 K for Windows (SPSS, Chicago, IL, USA). Data homogeneity of variances was analyzed by the Levene's test. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test post hoc test. Spearman's correlation was applied for trend analysis. The data presented in this study were expressed as mean ± standard deviation. The criterion of $p < 0.05$ was used for statistical significance.

Table 2. Primer sequence information.

Model	Gene	Primer Sequence (5'-3')	Accession No.
GH3	*Cyclophilin*	F: tctgagcactggggagaaag	M19533.1
		R: atgccaggacctgtatgctt	
	Tshβ	F: acagaacggtggaaataccg	NM_013116.2
		R: tctgtggcttggtgcagtag	
	Trα	F: tatcacttgtgagggctgca	NM_031134.2
		R: cacagcgatgcacttcttga	
	Trβ	F: atgttttgtgagctgccctg	J03933.1
		R: catgcccaggtcaaagatcg	
	Dio1[a]	F: gtggtggtggacacaatgcag	NM_021653
		R: ttgtagttccaagggccaggttta	
	Dio2	F: cagctttctcctagacgcct	NM_031720.3
		R: gcaaagtcaagaaggtggca	
	Fsh	F: acctggccatgatgaagtcg	NM_00100759
		R: caccagatccctggtgtagc	
	Erα	F: tgcctctggctaccattatgg	NM_012689.1
		R: tatgtccttgaatgcttctcttaaagaa	
	Erβ	F: tgagcaaagccaagagaacg	NM_012754.1
		R: ccagttgctctggactcaaggt	
H295R	*β-ACTIN*	F: cactcttccagccttccttcc	NM_001101
		R: aggtctttgcggatgtccac	
	StAR	F: gtcccaccctgcctctgaag	NM_000349
		R: catactctaaacacgaaccccacc	
	CYP 19	F: aggtgctattggtcatctgctc	NM_000103
		R: tggtggaatcgggtctttatgg	
	CYP 17	F: agccgcacaccaactatcag	NM_000414
		R: tcaccgatgctggagtcaac	

3. Results

3.1. Cytotoxicity test result from GH3 and H295R cells

WST-1 based colorimetric assay was performed for both GH3 and H295R cells in order to investigate cytotoxic effects from different concentrations of each thermal paper extracts. In GH3 cells, T3 exposure induced cell proliferation while BPA and BPA free thermal receipt extracts reduced the cell viability at the highest concentration (0.15 mg color developer/g receipt paper). The same trend occurred in H295R cells. For non-bisphenol thermal receipt paper, neither excess proliferation nor cytotoxicity was occurred.

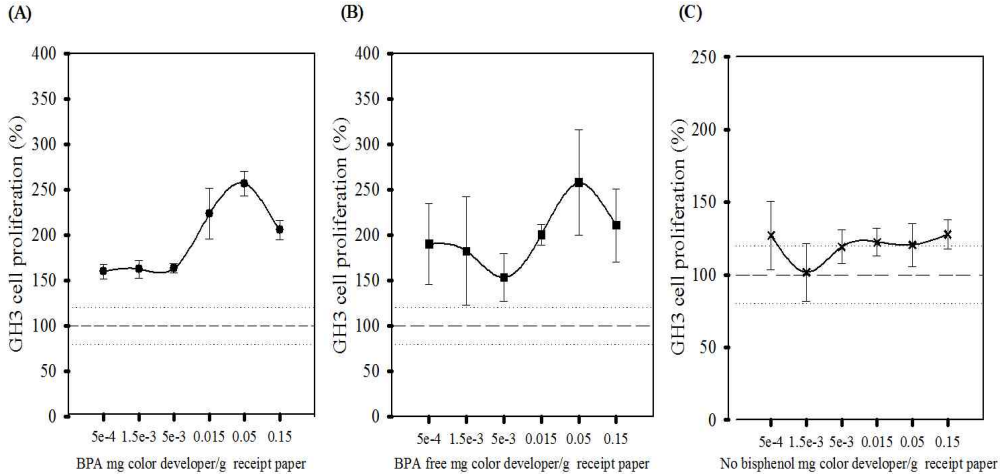

Figure 1. Preliminary range-finding tests of thermal receipt paper extracts. GH3 proliferations were measured following exposure to (A) BPA, (B) BPA free, and (C) non-bisphenol based thermal receipt paper extracts. The cell proliferation (%) was normalized to that of solvent control (0.1 % EtOH v/v). The error bar is the standard deviation of the technical replicates (n=5).

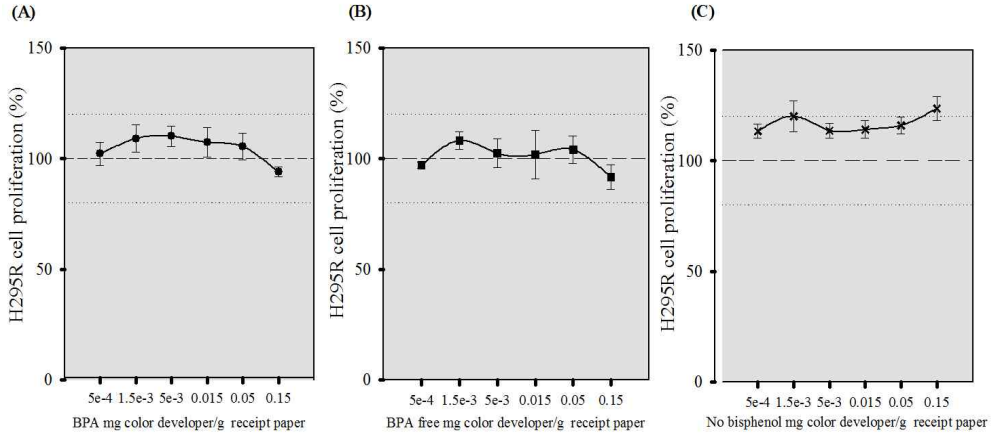

Figure 2. Preliminary range-finding tests of thermal receipt paper extracts. H295R cytotoxicity was measured following exposure to (A) BPA, (B) BPA free, and (C) non-bisphenol based thermal receipt paper extracts. The cell proliferation (%) was normalized to that of solvent control (0.1 % EtOH v/v). The error bar is the standard deviation of the technical replicates (n=3).

3.2. Gene transcriptional changes in GH3 cells

3.2.1. Pituitary-thyroid axis

All three paper extracts showed down regulated gene expressions in thyroid-stimulating hormone beta (*Tshβ*) in dose response manner (Figure 3A). BPA and BPA free thermal paper receipt paper extracts induced down-regulation in transcription levels significantly. T3 reduced transcriptional levels in both thyroid hormone receptors (*Trα* and *Trβ*) at its highest concentration, but all three extracts did not significantly affect those two genes (Figure 3B & C). Deiodinase type 1 (*Dio1*) gene expression levels was reduced in BPA exposed cells (Figure 3D). However, the gene expression of deiodinase type 2 (*Dio2*) was not affected from all three paper extractions (Figure 3E).

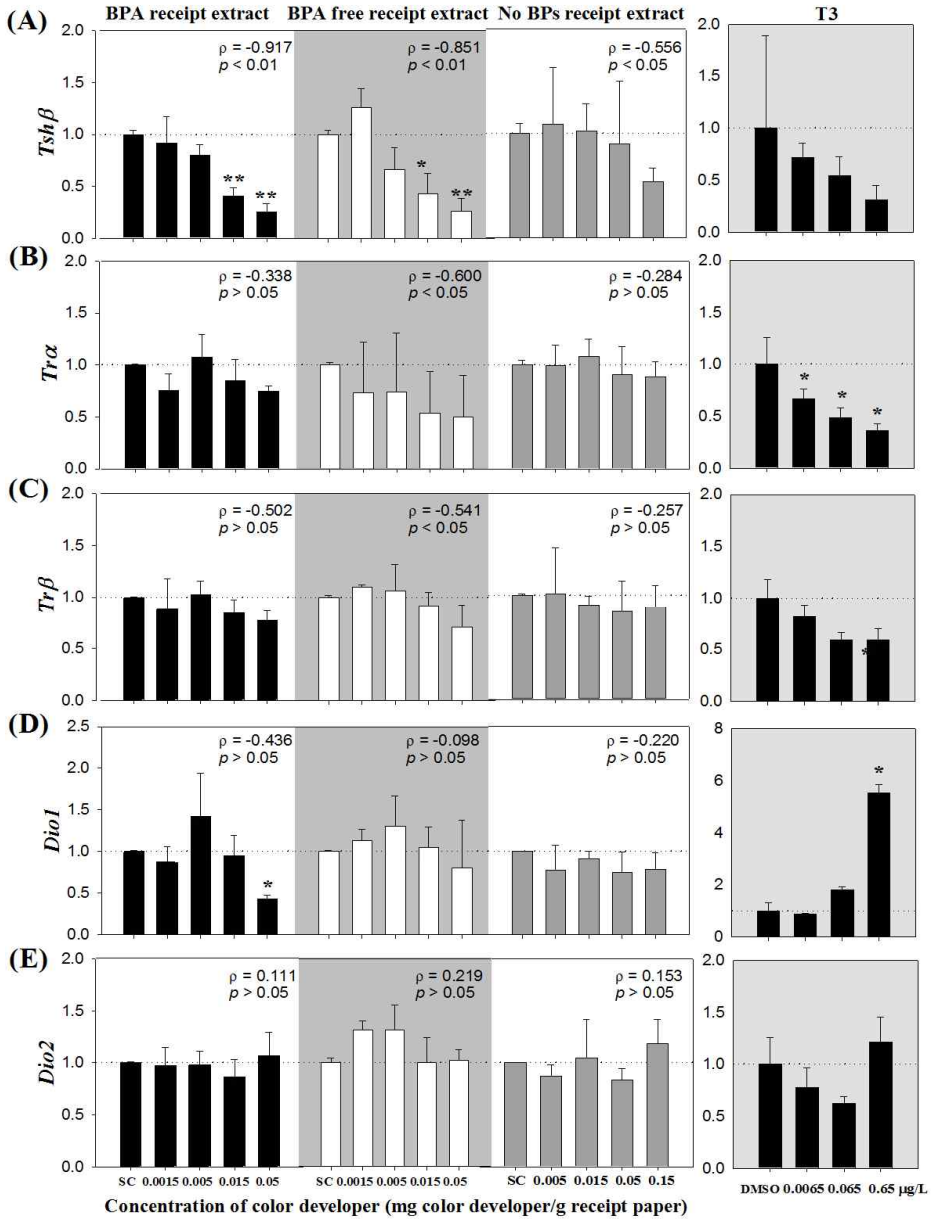

Figure 3. Thyroid hormone system related genes in GH3 cell line. Effects on (A) *Tshβ*, (B) *Trα*, (C) *Trβ*. (D)*Dio1,* and (E) *Dio2* gene transcriptions in GH3 cell line after exposure to 0, 0.0015, 0.005, 0.015, 0.05, or 0.15 mg developer/g receipt paper for 48 h. The error bar represents the standard deviation of three independent experiments (n=3). Asterisks (p* < 0.05) indicate significant difference compared to solvent control (SC, EtOH 0.1 %). The *ρ* and *p* values were determined based on Spearman's correlation.

3.2.2. Pituitary-gonad axis

The gene transcription levels for follicular stimulating hormones (*Fsh*), estrogen receptor alpha (*Erα*) and beta (*Erβ*) genes were analyzed (Figure 4). Cells were exposed to BPA and BPA free receipt extractions showed down regulations on *Fsh, Erα,* and *Erβ* (Figure 4A & 4B). Non-bisphenol receipt extract exposed cells had suppressed trend in *Fsh* gene expression. In addition, the gene expressions of *Erα* and *Erβ* were not significantly changed (Figure 4C).

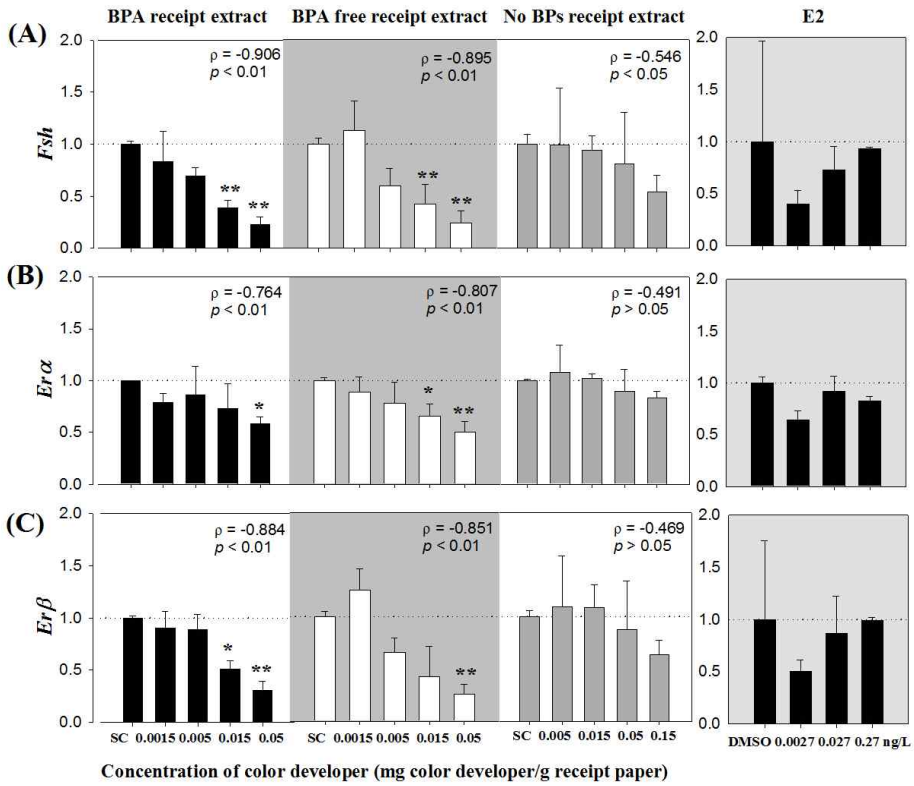

Figure 4. Sex hormone system related genes in GH3 cell line. Effects on (A) *Fsh*, (B) *Erα*, and (c) *Erβ* gene transcriptions in GH3 cell line after exposure to 0, 0.0015, 0.005, 0.015, 0.05, or 0.15 mg developer/g receipt paper for 48 h. The error bar represents the standard deviation of three independent experiments (n=3). Asterisks (p* < 0.05) indicate significant difference compared to solvent control (SC, EtOH 0.1 %). The ρ and p values were determined based on Spearman's correlation.

3.3. Hormone synthesis and related gene transcription in H295R cell

3.3.1. Hormone synthesis in H295R cell

All three thermal receipts significantly increased E2 production in H295R cells at 0.05 mg developer/g receipt paper for BPA receipt and BPA free receipt and 0.15 mg developer/g receipt paper for non-bisphenol receipt (Figure 5A). However, they did not affect the testosterone production. All three extracts showed different potency on E2 productions. BPA thermal receipt paper extract produced highest E2 amount at the highest concentration (0.05 mg developer/g receipt paper). BPA free thermal receipt paper increased E2 at is highest exposure concentration (0.05 mg developer/g receipt paper), yet the production amount was lower than BPA thermal receipt extract. Non-bisphenol thermal receipt extract also increased E2 at 0.15 mg developer/g receipt paper.

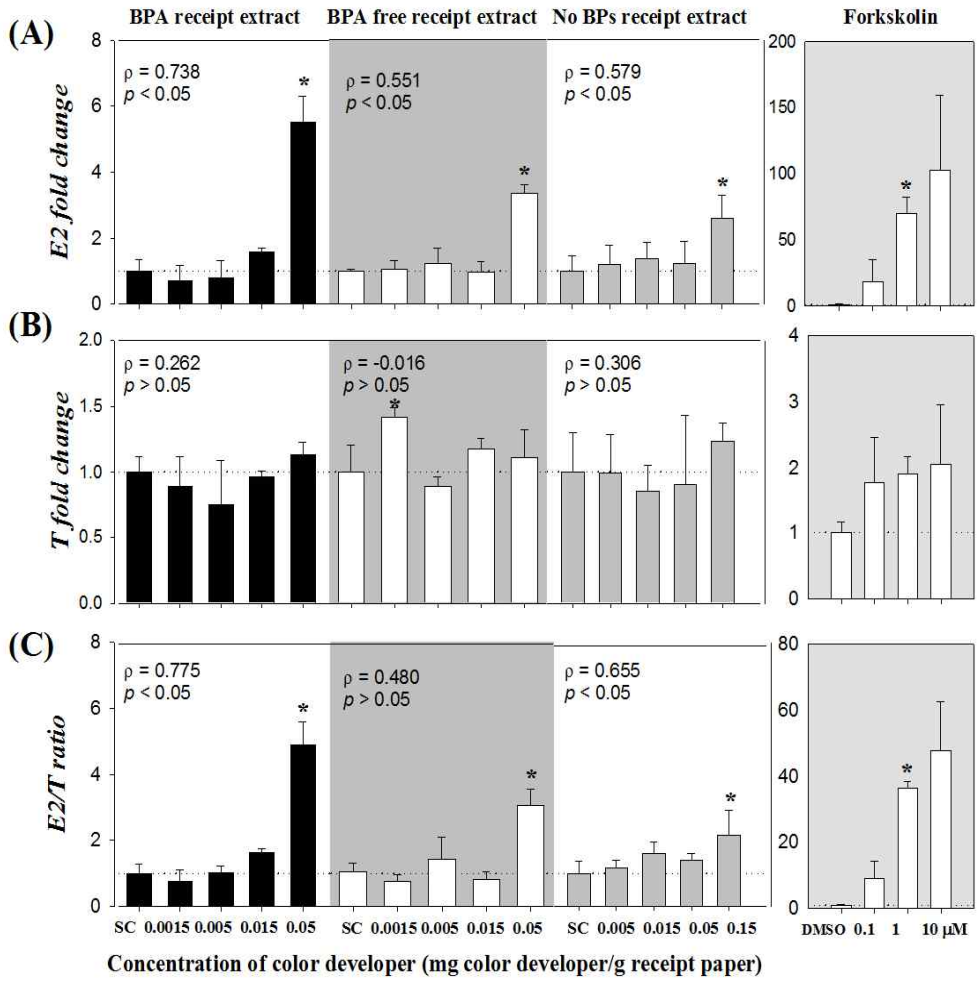

Figure 5. E2 and T level changes in H295R cells. Effects on (A) E2 (17β-estradiol), (B) T (Testosterone), and (c) E2/T ratio measured in H295R cell line after exposure to 0, 0.0015, 0.005, 0.015, 0.05, or 0.15 mg developer/g receipt paper for 48 h. The error bar represents standard deviation of three replicates (n=3), Asterisks (p* < 0.05) indicate significant difference compared to solvent control (SC, EtOH 0.1%). The *ρ* and *p* values were determined based on Spearman's correlation.

3.3.2. Steroidogenic gene expression changes

Three important steroidogenic genes involved in hormone sythesis such as *CYP19A1, CYP17A1,* and *StAR* were measured in H295R cell (Figure 6). None of genes were significantly changed their transcription levels after exposure to the extracts.

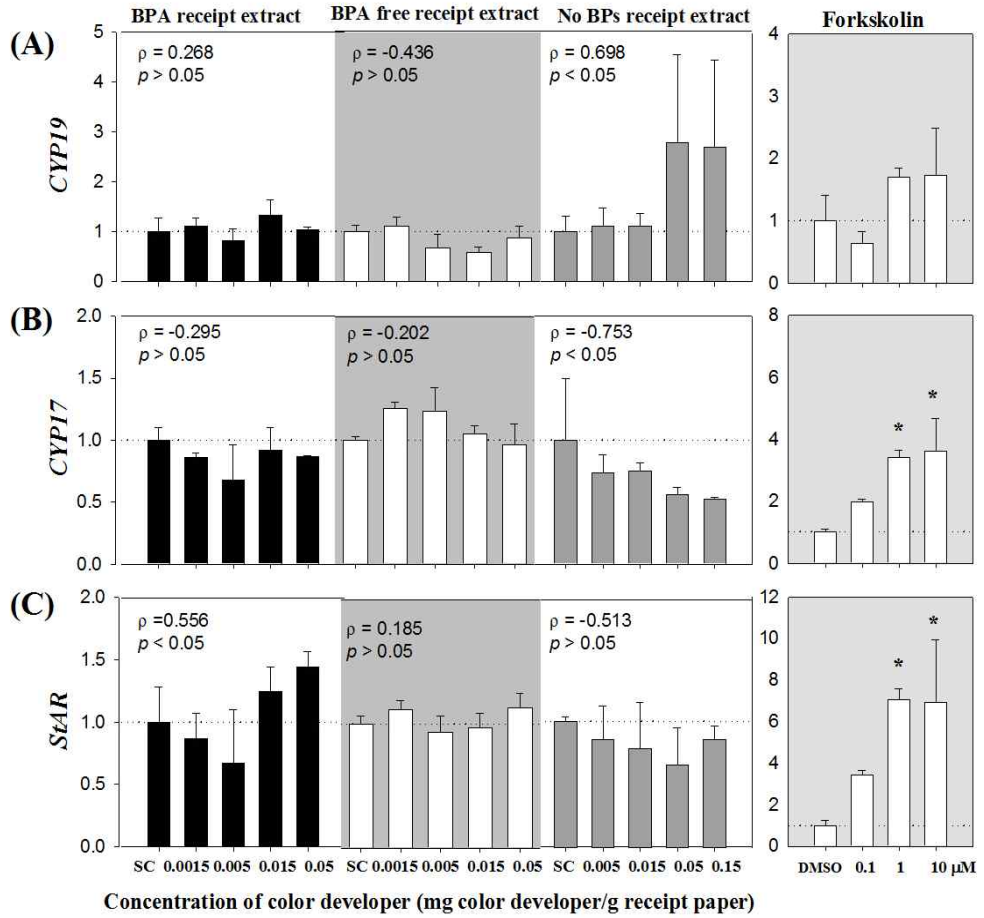

Figure 6. Sex hormone synthesis related genes in H295R cells Effects on (A) *CYP 19*, (B) *CYP17*, and (c) *StAR* gene transcription in H295R cell line after exposure to 0, 0.0015, 0.005, 0.015, 0.05, or 0.15 mg developer/g receipt paper for 48 h. The error bar represents standard deviation of three replicates (n=3), Asterisks (p* < 0.05) indicate significant difference compared to solvent control (SC, EtOH 0.1%). The ρ and p values were determined based on Spearman's correlation.

4. Discussion

In this study, we employed GH3 and H295R cell lines to screen the endocrine disruptive effects of different thermal receipt extracts in thyroid and sex hormone systems, respectively. The extracts are mixture of various chemicals applied on thermal receipt papers with color developer such as BPA and its structural or functional alternatives.

GH3 cell line from rat pituitary tumor is a standard model to test pituitary axis in thyroid hormone system (Kitamura et al., 2002). GH3 is sensitive to T3 treatment. Treatment of T3 induces down-regulation in *Tsh* gene in response of negative feedback. In this study, bisphenol applied thermal receipt papers showed down-regulated *Tshβ* gene expression, a similar to the observation from T3 exposed cell. The results are comparable to recent study which reported down-regulation of *Tshβ* gene level in GH3 cells exposed to BPA and its analogues (Lee et al., 2017). However, GH3 exposed to no bisphenol applied thermal receipt paper extract showed decreasing trend in *Tshβ* gene expression. Bisphenol compounds were not mimicking thyroid hormone because the discrepant patterns were observed in *Trα*, *Trβ*, and *Dio1* transcription levels (Figure 3). BPA and other bisphenols may impair thyroid hormone system in different mechanism such in nuclear hormone receptor binding (Moriyama et al., 2002; Sheng et al., 2012). In a previous study, BPA showed no detectable thyroid receptor binding activity in a yeast two-hybrid assay system (Kitagawa et al., 2003). However, another study proposed differebt mechanism of possible thyroid system alteration related to receptor binding. T3

binding receptors recruited steroid receptor coactivator-2 and enhanced gene expression whereas, un-liganded thyroid hormone receptors recruited nuclear hormone receptor corepressor and repressed transcription (Levy Bimbot et al., 2012).

Interestingly, BPA free receipt paper extracts led to down-regulated transcription trends in *Trα* and *Trβ* (Figure 3B & 3C). BPS is popular BPA replacement in thermal receipt paper industry ((Liao et al., 2012; Thayer et al., 2016; Björnsdotter et al., 2017 a). *Trα* and *Trβ* transcription level were significantly decreased in GH3 cells exposed to BPS at concentration from 0.1 mg/L (Lee et al., 2017). However, *Dio1* and *Dio2* gens were not similar to Lee et al. (2017). The effect may be compounded by other chemical compounds that present in the BPA free receipt paper extract.

GH3 cell lines are sensitive to T3, nevertheless, pituitary is the tissue involving in both thyroid and sex hormone production. In this study, transcriptional changes in *Fsh, Erα* and *Erβ* genes were observed together besides of thyroid axis. Bisphenol compound treated thermal receipt paper extracts showed decrease in *Fsh, Erα* and *Erβ* gene expressions. FSH is majorly regulated by gonadotropin-releasing hormone from hypothalamus with estrogen negative feedback, but this study was only focused on pituitary. In human study, the estrogen negative feedback occurs directly at the pituitary (Shaw et al., 2010). Another study observed estradiol reduced basal secretion of FSH in ewe's pituitary cells (Baratta et al., 2001). The weak estrogenic property of BPA and other bisphenol compound may cause the down-regulation of *Fsh* expression. *Erβ* from female ovariectomized rat pituitary were suppressed by

estrogen and its mRNA level were fell 40% (Schreihofer et al., 2000). GH3 exposed to progesterone (P) or P plus estrogen showed that *Erβ* expression was down regulated whereas no significant regulation activity was observed in *Erα* (Schreihofer et al., 2000). In this study, both estrogen receptors were suppressed from bisphenol compound thermal receipt paper extracts, but transcription of *Erβ* gene was more influenced than *Erα*. However, other GH3 cell study had reported opposite observation on *Erβ* from estradiol exposure, which showed mRNA level were increased by estradiol (Mitchner et al., 1999). Even though the literatures showed cacophony results on *Erβ,* estradiol alters pituitary function. The depression of *Erα* mRNA level may altered by other chemical compounds in the extracts.

The H295R result showed increased estrogenicity, e.g., E2 increase or weak decrease in testosterone production, which was similar to reports from other literature (Zhang et al., 2011; Goldinger et al., 2015; Feng et al., 2016). In H295R cells, all three extracts increased the E2 synthesis at the highest exposure level. Interestingly, the production amounts of E2 were varying. Cells exposed to BPA thermal receipt extract had highest production followed by that of BPA free thermal receipt extract. The non-bisphenol thermal receipt extract exposed cells produced significant amount of E2 compare to solvent control group, yet it was almost 2-fold lower than what BPA extract exposed cells were produced. However, testosterone levels were not affected from all three extracts exposures. In order to interpret the hormonal changes, we measured gene expressions related to sex hormone synthesis pathway (*CYP19*, *CYP17,* and *StAR*). However, we could not observe the transcriptional changes in the three major important genes. Zhang et al (2011)

reported that increased E2 in the cell medium was from inhibition of E2 metabolism rather than hormone synthesis activity. The three extracts may have different potency in hormone metabolism-related activity. Further study needs to focus on possible effects of metabolism, e.g., E2-sulfotransferase and E2-glucuronidase activity levels to understand the high E2 detection level.

Unfortunately, this study did not conduct a chemical analysis on the three thermal receipt paper samples and their extracts. We did not know what major chemical compound is causing toxic effects demonstrated in this study.

This study has many limitations and data gap. However, the results show that the thermal receipt paper may cause the alteration in thyroid or sex hormone systems. Further confirmation in vivo and long term exposure is warranted.

5. Conclusion

The concern of using BPA in thermal paper has been globally arising. Many chemists from different countries have reported BPA levels in thermal receipt papers. Chemicals that are structurally and functionally similar to BPA applied onto the thermal receipt papers as potential BPA replacements. However, it is hard to conclude that their adverse effects are less toxic than that of BPA from limited study results. This study has many limitations. Nevertheless, it should be highlighted that the BPA free thermal receipt papers are composing potential endocrine disruption effects.

References

Aghajanpour-Mir, S.M., Zabihi, E., Akhavan-Niaki, H., Keyhani, E., Bagherizadeh, I., Biglari, S., Behjati, F., 2016. The Genotoxic and Cytotoxic Effects of Bisphenol-A (BPA) in MCF-7 Cell Line and Amniocytes. International Journal of Molecular and Cellular Medicine 5, 19-29.

Baratta, M., West, L., Turzillo, A., Nett, T., 2001. Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells. Biology of reproduction 64, 714-719.

Biedermann, S., Tschudin, P., Grob, K., 2010. Transfer of bisphenol A from thermal printer paper to the skin. Analytical and Bioanalytical Chemistry 398, 571-576.

Björnsdotter, M.K., de Boer, J., Ballesteros-Gómez, A., 2017 a. Bisphenol A and replacements in thermal paper: A review. Chemosphere 182, 691-706.

Bjornsdotter, M.K., Jonker, W., Legradi, J., Kool, J., Ballesteros-Gomez, A., 2017 b. Bisphenol A alternatives in thermal paper from the Netherlands, Spain, Sweden and Norway. Screening and potential toxicity. The Science of the total environment 601-602, 210-221.

Bradley, E.L., Honkalampi-Hamalainen, U., Weber, A., Andersson, M.A., Bertaud, F., Castle, L., Dahlman, O., Hakulinen, P., Hoornstra, D., Lhuguenot, J.C., Maki-Paakkanen, J., Salkinoja-Salonen, M., Speck, D.R., Severin, I., Stammati, A., Turco, L.,

Zucco, F., von Wright, A., 2008. The BIOSAFEPAPER project for in vitro toxicity assessments: preparation, detailed chemical characterisation and testing of extracts from paper and board samples. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 46, 2498-2509.

Burridge, E., 2008. Chemical profile: bisphenol A., ICIS Chemical Business

Fan, R., Zeng, B., Liu, X., Chen, C., Zhuang, Q., Wang, Y., Hu, M., Lv, Y., Li, J., Zhou, Y., Lin, Z.Y., 2015. Levels of bisphenol-A in different paper products in Guangzhou, China, and assessment of human exposure via dermal contact. Environmental science. Processes & impacts 17, 667-673.

Feng, Y., Jiao, Z., Shi, J., Li, M., Guo, Q., Shao, B., 2016. Effects of bisphenol analogues on steroidogenic gene expression and hormone synthesis in H295R cells. Chemosphere 147, 9-19.

Geens, T., Aerts, D., Berthot, C., Bourguignon, J.-P., Goeyens, L., Lecomte, P., Maghuin-Rogister, G., Pironnet, A.-M., Pussemier, L., Scippo, M.-L., 2012. A review of dietary and non-dietary exposure to bisphenol-A. Food and chemical toxicology 50, 3725-3740.

Goldinger, D.M., Demierre, A.-L., Zoller, O., Rupp, H., Reinhard, H., Magnin, R., Becker, T.W., Bourqui-Pittet, M., 2015. Endocrine activity of alternatives to BPA found in thermal paper in Switzerland. Regulatory Toxicology and Pharmacology 71, 453-462.

Hehn, R.S., 2015. NHANES data support link between handling of thermal paper receipts and increased urinary bisphenol a excretion. Environmental Science & Technology 50, 397-404.

Kuruto-Niwa, R., Nozawa, R., Miyakoshi, T., Shiozawa, T., Terao, Y., 2005. Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. Environmental Toxicology and Pharmacology 19, 121-130.

Lassen, C., Brandt, U.K., 2011. Migration of bisphenol A from cash register receipts and baby dummies. Environmental Protection Agency.

Liao, C., Kannan, K., 2011. Widespread occurrence of bisphenol A in paper and paper products: implications for human exposure. Environmental science & technology 45, 9372-9379.

Liao, C., Liu, F., Kannan, K., 2012. Bisphenol s, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol a residues. Environ Sci Technol 46, 6515-6522.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 25, 402-408.

Mendum, T., Stoler, E., VanBenschoten, H., Warner, J.C., 2011. Concentration of bisphenol A in thermal paper. Green Chemistry Letters and Reviews 4, 81-86.

Mitchner, N.A., Garlick, C., Steinmetz, R.W., Ben-Jonathan, N., 1999. Differential regulation and action of estrogen receptors α and β in GH3 cells. Endocrinology 140, 2651-2658.

Ndaw, S., Remy, A., Jargot, D., Robert, A., 2016. Occupational exposure of cashiers to Bisphenol A via thermal paper: urinary biomonitoring study. International archives of occupational and environmental health 89, 935-946.

Östberg T, N.E., 2010. Bisfenol A in Svenska Kvitton. Analysresultat. .

Rocha, B.A., Azevedo, L.F., Gallimberti, M., Campiglia, A.D., Barbosa Jr, F., 2015. High levels of bisphenol A and bisphenol S in Brazilian thermal paper receipts and estimation of daily exposure. Journal of Toxicology and Environmental Health, Part A 78, 1181-1188.

Russo, G., Barbato, F., Grumetto, L., 2017. Monitoring of bisphenol A and bisphenol S in thermal paper receipts from the Italian market and estimated transdermal human intake: A pilot study. Science of The Total Environment 599-600, 68-75.

Schreihofer, D.A., Stoler, M.H., Shupnik, M.A., 2000. Differential expression and regulation of estrogen receptors (ERs) in rat pituitary and cell lines: estrogen decreases ERalpha protein and estrogen responsiveness. Endocrinology 141, 2174-2184.

Shaw, N.D., Histed, S.N., Srouji, S.S., Yang, J., Lee, H., Hall, J.E., 2010. Estrogen Negative Feedback on Gonadotropin Secretion: Evidence for a Direct Pituitary Effect in Women. The Journal of clinical endocrinology and metabolism 95, 1955-1961.

Terasaki, M., Shiraishi, F., Fukazawa, H., Makino, M., 2007. Occurrence and estrogenicity of phenolics in paper-recycling process water: pollutants originating from thermal paper in waste paper. Environmental toxicology and chemistry 26, 2356-2366.

Thayer, K.A., Taylor, K.W., Garantziotis, S., Schurman, S.H., Kissling, G.E., Hunt, D., Herbert, B., Church, R., Jankowich, R., Churchwell, M.I., Scheri, R.C., Birnbaum, L.S., Bucher, J.R., 2016. Bisphenol A, Bisphenol S, and 4-Hydroxyphenyl 4-Isoprooxyphenylsulfone (BPSIP) in Urine and Blood of Cashiers. Environmental health perspectives 124, 437-444.

U.S. Environment Protection Agency. 2015. Bisphenol A Alternative in Thermal Paper, Final Report.

Vandenberg, L.N., Chahoud, I., Heindel, J.J., Padmanabhan, V., Paumgartten, F.J., Schoenfelder, G., 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environmental health perspectives, 1055-1070.

Vinggaard, A.M., Korner, W., Lund, K.H., Bolz, U., Petersen, J.H., 2000. Identification and quantification of estrogenic compounds in recycled and virgin paper for household use as determined by an in vitro yeast estrogen screen and chemical analysis. Chemical research in toxicology 13, 1214-1222.

von Goetz, N., Pirow, R., Hart, A., Bradley, E., Poças, F., Arcella, D., Lillegard, I.T.L., Simoneau, C., van Engelen, J., Husoy, T., Theobald, A., Leclercq, C., 2017. Including non-dietary sources into an exposure assessment of the European Food Safety Authority: The challenge of multi-sector chemicals such as Bisphenol A. Regulatory Toxicology and Pharmacology 85, 70-78.

Yamasaki, K., Noda, S., Imatanaka, N., Yakabe, Y., 2004. Comparative study of the uterotrophic potency of 14 chemicals in a uterotrophic assay and their receptor-binding affinity. Toxicol

Lett 146, 111-120.

Zhang, D.H., Zhou, E.X., Yang, Z.L., 2017. Waterborne exposure to BPS causes thyroid endocrine disruption in zebrafish larvae. PLoS One 12, e0176927.

Zhang, X., Chang, H., Wiseman, S., He, Y., Higley, E., Jones, P., Wong, C.K., Al-Khedhairy, A., Giesy, J., Hecker, M., 2011. Bisphenol A disrupts steroidogenesis in human H295R cells. Toxicological Sciences, kfr061.

국문 초록

영수증의 내분비계 교란 스크리닝 연구

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Bisphenol A는 약한 에스트로겐 성질을 가진 화학물질로 알려져 있으나 여전히 많은 산업과 소비자 제품에 사용되고 있는 물질이다. 음식물 섭취를 통한 BPA 노출을 제외하면 영수증 접촉은 주요 BPA노출 원인이다. BPA는 감열지에서 색을 내는데 도움을 주는 현색제로 사용되고 있다. 하지만, BPA의 독성으로 인해 화학구조적으로 비슷하거나 기능이 비슷한 대체물질이 감열지에 사용되고 있다. 그러나 이러한 대체물질에 대한 독성정보는 BPA 독성정보에 비해 부족한 실정이다.

본 연구에서는 서로 다른 현색제가 입혀진 세 종의 감열영수증(BPA, BPA free, 무 비스페놀류 감열지 영수증)에 대한 독성을 비교하고자 하였다. GH3와 H295R 세포주에 감열영수증 추출액을 노출시켜 갑상선과 성선 호르몬에 미치는 독성영향을 비교하였다. 현색제의 양은 총 영수증 무게의 1 %에 해당한다고 기존 논문을 통해 가정하였다. GH3와 H295R 세포 모두 0, 0.0015, 0.005, 0.015, 0.05, 0.15 mg 현색제/g 감열지로 계산하여 0.1 % ethanol의 세포 미디어에 48시간 노출 시켰다. BPA와 BPA free 영수증 추출액은 GH3 세포에서 감상샘자극호르몬 (*Tsh*) 유전자 발현을 저해하였다. 하지만, 다른 유전자인 *Trα, Trβ, Dio1, Dio2* 에서는 발현량의 차이가 보이지 않았다. GH3 세포에서 성호르몬과 관련해서는

소낭자극호르몬(*Fsh*), 에스트로젠 수용체 α 와 β (*Erα*와 *Erβ*) 유전자 발현 또한 BPA, BPA free 감열지 추출액 노출 후 감소하였다. 무 비스페놀류 감열지 영수증에 노출된 세포는 유전자적 수준의 변화가 관찰되지 않았다. H295R 세포주에서는 BPA, BPA free, 무 비스페놀 영수증 추출액에 노출된 세포에서 에스트로겐 호르몬 양이 고농도군 (BPA 와 BPA free영수증: 0.05 mg/g; 무비스페놀: 0.15 mg/g)에서 유의하게 증가하였지만 테스토스테론 호르몬 양은 변화가 없었다.

본 연구에서는 세 종의 다른 현색제가 입혀진 감열영수증의 내분비계 교란영향을 확인 할 수 있었다. 하지만 본 연구의 한정적인 정보로 비스페놀류가 아닌 현색제가 사용된 영수증이 비스페놀류 현색제 감열영수증보다 안전하다고 판단하기는 어렵다. 그럼에도 불구하고, 본 연구는 비스페놀류 현색제가 입혀진 감열영수증의 독성을 확인 할 수 있는 연구이다. 추후 동물 모델을 이용하여 영수증 독성 확인이 필요 될 것으로 사료된다.

주요어: 감열지, 영수증. BPA. BPA free, 갑상선 호르몬, 성선호르몬, 대체물질

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